J. Clin. Chem. Clin. Biochem.
Vol. 15, 1977, pp. 271–274

A Discrete Multichannel Procedure for the Chemical Determination of Urinary Estrogens during Pregnancy

By *I. M. Penttilä, H. Jokela, E. Puhakainen, S. Nummi, T. Rantanen* and *A. J. Viitala*

The Department of Clinical Chemistry and the Clinic of Obstetrics and Gynaecology, Kuopio University Central Hospital, Kuopio, Finland

(Received July 19/December 20, 1976)

Summary: A colorimetric method, based on the *Kober* reaction, was adapted for the determination of urinary estrogens during pregnancy. The method yields final volumes for direct measurements by a multichannel computer-controlled photometer. The speed of the analysis was increased so that 100 samples can easily be completed by one laboratory technician as triplicate analyses during one working day. A satisfactory accuracy and precision of the method were also achieved. The values were determined using urine samples from 1097 apparently healthy pregnant women.

Eine selektive Vielkanalmethode für die chemische Bestimmung von Östrogenen im Harn von Schwangeren

Zusammenfassung: Eine semiautomatische kolorimetrische Methode zur Bestimmung von Östrogenen im Harn von Schwangeren wurde so modifiziert, daß die definitiven Volumina mit einem Computer-kontrollierten Multikanal-photometer gemessen werden konnten. Die Leistungsfähigkeit der Methode wurde so gesteigert, daß eine Laborantin während eines Arbeitstages 100 Proben mit drei parallelen Analysen mit einer *Allen*-Korrektur durchführen kann. Eine genügende Präzision wurde erreicht. Die Werte wurden im Harn von 1097 gesunden Schwangeren bestimmt.

Introduction

The determination of urinary estriol during pregnancy has been widely used for monitoring foetal welfare (1–5). However, clinical use of the chemical determination of estriol is restricted by the complexity and slowness of the method. Since estriol accounts for up to 90 per cent of the total estrogens in urine during late pregnancy (6, 7), the simpler determination of total estrogens rather than estriol has been used in routine daily work (8–14) in manual or automated procedures. The simplification and automation of estrogen assay have been shown to cause problems since some other urine components such as glucose (9, 15, 16), other saccharides and aldehydes (15), and some medicaments (9) react with the *Kober* reagent. The more accurate methods, which include several extractions, a number of absorbance measurements for parallel analyses and an *Allen* correction (17) to eliminate the effect of urinary non-estrogenic components, are cumbersome and time-consuming when manual laboratory equipment is used.

In the present paper a rapid and accurate method is described for the determination of urinary total estrogens during pregnancy using a modification of *Ittrich*'s procedure (10) using a System Olli 3000 Analyzer, a discrete multichannel analyzer connected on-line to the central processor unit of the laboratory (18). Our modification of the Olli 3000 multichannel photometric procedure allows complex manual procedures to be carried out on a large scale without decreasing the number of steps in the method. The reference values during pregnancy are also presented.

Materials and Methods

Materials

The analyses for reference values were performed for 1097 apparently healthy pregnant women. Twenty four hour urine samples were collected for urine analysis during the day preceding visits to the out-patient department. In most cases the analyses were performed on the day of urine collection.

Determination of total estrogens

The determination of total estrogens in pregnancy urine was performed using a modification of *Ittrich*'s procedure (19) as described by *Rourke* et al. (10). This method was modified for performance with an Ultrolab dispenser (LKB, Bromma,

Sweden) and a photometer of the System Olli 3000 Analyzer (Ollituote Oy, Kivenlahti, Finland) for large-scale sample analysis.

Procedure

900 µl of the well-mixed urine samples was diluted with 900 µl of 1.0 mol/l HCl (p. a. Merck) in a 15 ml extraction tube. Both standards (200 µmol/l) and analyses were prepared in triplicate. 1.5 g of crystalline NaCl (p. a. Merck) and 4.66 ml of ethyl acetate (p. a. Merck) were added to each tube. After vigorous shaking (2 min) the tubes were centrifuged for 10 min at 2000 *g*. The tubes were then left at room temperature for 10 min, after which 800 µl aliquots of the upper ethyl acetate phases were transferred to clean extraction tubes. The solutions were evaporated to dryness in a boiling water bath, thereby avoiding overheating. After cooling, 500 µl of distilled water was added to each tube to dissolve the residues. Then 1.0 ml of cool colour reagent (27,8 g/l of hydroquinone in H_2SO_4) was added to the tubes with occasional shaking. Finally the tubes were shaken vigorously using a Vortex mixer and placed in a boiling water bath for 40 min. After heating the tubes were chilled in an ice bath and 2.0 ml of distilled water was added, and the tubes chilled once again. Then 2.0 ml of cool extraction solution (20 g/l 4-nitrophenol in tetrachloroethane) was added and the tubes were vigorously shaken using a Vortex mixer. After centrifuging at 2000 *g* for 10 min the aqueous phases were discarded using a water jet jump and the lower organic phases were poured into cuvettes and measured without delay. If the tubes were measured using a normal photometer with a through-flow cuvette all volumes were doubled.

The absorbances of both standards and samples were measured against 4-nitrophenol solution in tetrachloroethane using either a Beckman B spectrophotometer with a through-flow cuvette (1 ml) (Beckman Instruments, Inc., Fullerton, USA) or a System Olli 3000 photometer. The Olli photometer (20) is capable of measuring absorbances from 24 parallel cuvettes in a cuvette block. The measurement time for one block is five seconds. Three wavelengths were used to perform an *Allen* correction (17) as follows: $A_{corr.} = A_{540} - 1/2\,(A_{570} + A_{510})$ (Beckman). The original interference filters of the Olli photometer with bandwidths from 9 to 11 nm were used; their wavelengths were 573.5, 542.5 and 510.3 nm.

For the final results an *Allen* correction (17) was first performed for every tube, then the two closest corrected absorbance values were selected and the mean values were used to calculate the results as follows:

$$\text{dU-Estrogens: } \frac{A_{corr.\ of\ sample}}{A_{corr.\ of\ stand.}} \times \text{standard value} \times \text{1000/urine vol.}$$

(µmol) (µmol/l) (ml)

The necessary calculations were performed by a Sony Sobax calculator (Sony Co., Japan) or by the central processor unit of the laboratory connected on-line to the Olli photometer (18).

Determination of estriol

The determination of estriol in urine during pregnancy was performed using the method described by *Beling* (21). The analyses were perfomed by "Kansanterveyslaboratorioiden keskuslaboratorio" (Central Laboratory of the National Health Laboratories, Helsinki, Finland).

Results and Discussion

We used a modification of *Ittrich*'s procedure (19) described by *Rourke* et al. (10) as a manual procedure for the determination of estrogens in urine during pregnancy. When the number of analyses in our laboratory increased it became necessary to develop a method which would give a larger-scale performance than the manual method. Other efforts were directed at increasing the precision of the method and obtaining the valid reference values for the method used.

Development of the method

The extraction of the estrogens from acidified urine in the presence of NaCl was performed in a slightly modified form according to *Rourke* et al. (10). During the colour development stage the ratio of water to sulphuric acid was kept at 1:2. If the sulphuric acid content is increased to over 67 per cent to give the most favourable reaction condition for estriol (22), the colour extraction stage cannot be performed as readily due to the greater volume of distilled water needed for proper separation of the phases. The colour extraction into 2.0 ml of tetrachloroethane containing 4-nitrophenol was made from an acidic aqueous solution of 3.5 ml. A volume of tetrachloroethane more than one half of the water volume is reported by *Salokangas & Dulbrook* (23) to be adequate.

At 542.5 nm the corrected absorbance value after the *Allen* correction (17) was found to be 91 per cent of the maximum at 537 nm. The method was found to give a linear response up to a concentration of 300 µmol/l. Comparing this with the other method (10, 19, 24) it seems that the small differences between the wavelengths used did not affect the linearity of the method.

Performing the analysis

As presented in table 1, the time needed for the photometric measurements and calculations for 100 samples including standards is about 18 minutes. This means that 927 absorbance measurements and 309 *Allen* corrections (17) must be done before the final results, in µmol/l and µmol/24 h, can be calculated. One laboratory technician

Tab. 1. The time needed for absorbance measurements and calculations when 100 analyses of urinary total estrogens were performed by one technician in triplicate using different instrumentation methods. The photometric time includes 927 absorbance measurements and the calculation period consists of the load of absorbances to the calculator or the transfer of absorbances to the computer in addition to the calculation time itself.

Instrumentation	Time [min] Photometer	Calculation	Total
Beckman B photometer Sony Sobax calculator	280	40	320
Olli 3000 photometer Sony Sobax calculator	13	40	53
Olli 3000 photometer on-line to CPU of laboratory	13	5	18

is capable of performing 100 analyses in triplicate during a normal eight-hour working day. This is a good performance when compared with other corresponding methods (8, 9, 10, 11, 12). Table 1 also shows how to save the cost of one technician's daily labour when developing the measurement and calculation parts of the manual method used previously.

Accuracy and precision of the method

The accuracy of the method was tested by adding estriol to urine in various concentrations. It was shown that the mean recovery of estriol added was 84.9 per cent (range 78–97 per cent). These results agree well with previous results from the extraction of estrogens from acidified urine (8, 10, 11, 12).

Comparing the values found using this method with the estriol values found by the method of *Beling* (21) shows a close correlation (fig. 1) between the estrogen and estriol content of urine. The mean estriol excretion into urine during the last three months of pregnancy was found to be 77.2 per cent of total estrogens, as found earlier (6, 7).

The precision of photometer measurement is quite good; measurements using the same cuvette in 24 positions of the block gave a variation coefficient of 1.6 per cent for a sample of 85.5 μmol/l. It was found that if the *Allen* correction (17) was made for the mean absorbance values of the two closest values from three measurements, the variation coefficient was regularly higher. Therefore it was not possible to reduce the number of *Allen* corrections for the calculations. The usual variation coefficient within a day was 4.1 per cent, while the day to day variation over one month (n = 24) was 6.1 per cent for a pooled sample of 83.5 μmol/l.

Values

Values for the daily urinary excretion of estrogens during the 25th and 40th weeks of pregnancy using the present method are presented in figure 2. Because of the positive skew distribution the mean values ± 2x S. D. were calculated as a lognormal distribution. The mean curve shows a gradual increase in excretion during pregnancy. There is, however, a slight tendency to a slower increase in excretion after the 38th week of pregnancy than before it, which could be due to the reported lack of increase in estrone and estradiol-17β excretion during the last weeks of pregnancy (24). The reference values presented are in good agreement with those reported earlier (8, 10).

Acknowledgement

The authors would like to thank Mrs. *Hanna-Maija Niiranen* for her valuable assistance during the development of the method.

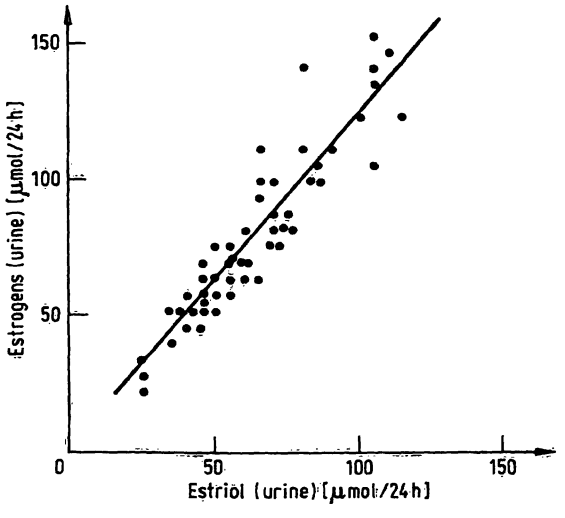

Fig. 1. The correlation of the estriol excretion measured by a specific method (21) with the excretion of estrogens measured by the present method (y = 2.41 + 1.26x; r = 0.852). The mean value (± 1 S. D.) was 61.4 ± 23.1 μmol/24 h for estriol and 79.5 ± 31.5 μmol/24 h for estrogens. All 53 samples were collected from subjects during the last trimester of pregnancy.

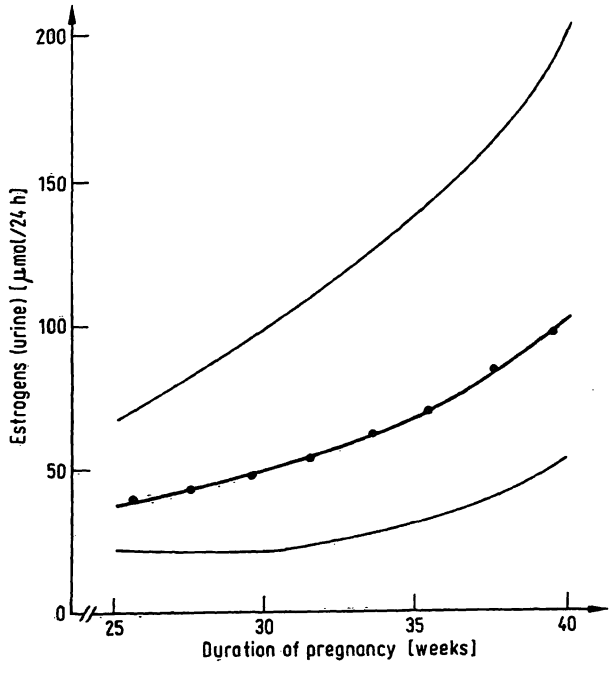

Fig. 2. The values for estrogen excretion in daily urine are based on the determination of values from 1097 apparently healthy pregnant women. The values were calculated on the basis of a lognormal distribution. The mean values are shown as dotted lines; 99.6 per cent of the material remains within the upper and lower limits.

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PD Dr. Ilkka Penttilä
Dept. Clin. Chem.
University Hospital
SF-70210 Kuopio 21